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How alterations in the Cdt1 expression leads to gene amplification in Breast Cancer

PRINCIPAL INVESTIGATOR:
Kenta Terai M.D., Ph.D.

CONTRACTING ORGANIZATION:
University of Virginia
Charlottesville, VA 22904

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Kenta Terai

Email: kt5m@virginia.edu

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14. ABSTRACT
We examined the effects of Cdt1 for gene amplification by using inducible system. We Obtained and validated reagents for inducible Cdt1-expression-system. Furthermore, we identified appropriated dose of MTX for testing gene amplification
Cdt2 is a key molecule for inhibiting Cdt1 activity. The depletion of Cdt2 induces DNA re-replication in several cell lines. During experiment, we Identified a reduction of PCNA monoubiquitination in Cdt2 depleted MCF7 cells. Exogenous Cdt2 rescued the effect of si-Cdt2. Mutant of Cdt2 indicated that binding Cdt2 to Cul4/DDB1 was required for PCNA monoubiquitination. Furthermore, we identified that CRL4Cdt2 complex promotes PCNA-dependent translesion DNA synthesis.

15. SUBJECT TERMS
Breast Cancer, PCNA, Cdt2, Re-replication, Genomic Instability

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Introduction:

Gene amplification is frequently observed in not only breast cancer but also in other malignant tumors. Overexpression and amplification of HER2/neu are reported in 25-30% of breast cancers. Patients with breast cancers that over-express HER2 have an aggressive form of the disease with poor prognosis. The number of sites and extent of gene amplification also indicate genetic instability of the cancer and predict a poor prognosis. Chromosomal replication origins are licensed by loading the Minichromosome maintenance 2–7 proteins (MCM2-7) complexes. Cdt1 is critical for loading MCM2-7, but it is inactivated by binding geminin and by ubiquitin mediated degradation after the onset of S phase so that origins are not licensed a second time in the cell cycle. Previous studies report that cells expressing excess Cdt1 re-replicated their DNA. Cdt1 is over-expressed in human breast cancer cells and over-expression of Cdt1 in mice predisposes them to tumorigenesis.

Cdt1 is degraded by ubiquitin-mediated proteolysis soon after the onset of S phase. There are two pathways for the degradation of Cdt1. In S and G2 phases, Cdt1 is phosphorylated by Cyclin E/A dependent CDK2 kinase, and the phosphorylated Cdt1 interacts with Skp2 to be targeted for degradation by SCF-mediated ubiquitination that depends on Skp1 and Cul1. Cul4A/DDB1 mediated ubiquitination is a second, CDK2-independent pathway of ubiquitinating Cdt1 during DNA replication. Indeed, Cdt1 has two signaling regions for degradation: a signal in residues 1-18 of Cdt1 for the Cul4A/DDB1 pathway and a cyclin-binding Cy-motif (around residue 62) and T29 signal for the Skp2-SCF pathway. Intriguingly, Cul4 is frequently amplified in breast cancers (Schindl et al., 2007) suggesting that deregulation of Cdt1 stability may be common in breast cancers. However, there is no report yet on the levels of expression of Cdt1 in breast cancer.

The CRL4Cdt2 E3 ubiquitin ligase complex is a member of the cullin-RING family that was recently shown to promote the polyubiquitination and degradation of the replication licensing factor Cdt1(Arias and Walter, 2006; Higa et al., 2006; Jin et al., 2006; Senga et al., 2006). The E3 ubiquitin ligase complex consists of Cul4A or Cul4B, DDB1 (damage-specific DNA binding protein 1), the RING-finger protein ROC1 and the DCAF substrate recognition factor, and the WD40 protein, Cdt2. Additional substrates for the CRL4Cdt2 ubiquitin ligase complex have been recently described including p21 (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008), the *C. elegans* polymerase eta (Kim and Michael, 2008) and the *D. melanogaster* E2f1 transcription factor (Shibutani et al., 2008). Notably, most identified CRL4Cdt2 substrates require their interaction with PCNA for their polyubiquitination. The exact role of PCNA is not clear but may lie in the recognition of substrate via Cdt2.

Body:

Validating Cdt1 expression by using Doxcycline in a dose-dependent manner.

Recently, a Doxcycline-inducible Cdt1-expression-system has reported by using Doxcycline (Liontos et al., 2007). We obtained these cell lines and tested. As shown in Figure 1A, Cdt1 was nicely induced with 0.1 ug/ml of Doxcycline in U2OS colon cancer cell lines. Since this system can be transferred to other cell lines, we will establish Cdt1 expressing in breast cancer cell lines.

Testing the toxicity of MTX in breast cancer cell lines.

For Aim 3, I will test whether moderate over expression of Cdt1 promotes DNA amplification. Methotrexate (MTX) inhibits the DHFR gene, and a common method by which

breast epithelial cells become resistant to MTX is through the amplification (and overexpression) of DHFR. To identify how much dose of MTX is required for inducing gene amplification, I tested the toxicity of MTX in breast cancer cell lines (Fig. 1B). Cell growth was inhibited by between 10-50 nM of MTX. Under these conditions, I will test whether Cdt1 over-expression promotes gene amplification of DHFR and resistance to MTX.

Identifying a reduction of PCNA monoubiquitination in Cdt2 depleted MCF7 cells.

Cdt2 is a key molecule for inhibiting Cdt1 activity. The depletion of Cdt2 induces DNA re-replication in several cell lines. Thus, we depleted Cdt2 in MCF7 breast cancer cell lines (Fig. 2). Notably, we identified that Cdt2 depleted cells showed lower level of PCNA monoubiquitination without UV treatment.

The monoubiquitination of PCNA is one of the best understood example of protein monoubiquitination at a conserved lysine residue (K164 in human PCNA) in response to DNA damage, increases its affinity for members of the Y-family of DNA bypass polymerases, and allows DNA synthesis across DNA lesions (Andersen et al., 2008). PCNA monoubiquitination in response to DNA damage is dependent on the activity of the ubiquitin conjugating E2 enzyme Rad6 and the ligase activity of the Rad18 E3 ubiquitin ligase (Hoege et al., 2002; Kannouche et al., 2004) and cells deficient of either of these enzymes are extremely sensitive to a variety of DNA damaging agents (Andersen et al., 2008). Intriguingly, several reports demonstrated the presence of residual monoubiqutinated PCNA in Rad18-deficient cells, indicating that Rad18-independent ubiquitin ligase activity exists to promote PCNA monoubiquitination (Brun et al., 2008; Huang et al., 2006; Parris and Seidman, 1992). Thus, we tested whether Cdt2 and CRL4 complex promote PCNA monoubiquitination.

The depletion of Cdt2 reduced basal level of PCNA mono-ubiquitination.

Previously, it has been reported that the induction of PCNA monoubiqutinatiton by depleting USP1, the enzyme responsible for deubiquitinating PCNA, is independent from Rad18 (Simpson et al., 2006). Thus, we hypothesized that knocking down Cdt2 may affect the basal level of PCNA monoubiqutination under USP1-depleted condition. As expected, treatment of U2OS cells with si-USP1 promoted PCNA monoubiquitination (Figure 3A). Furthermore, treatment of Rad18 siRNA reduced PCNA monoubiquitination in the absence of USP1. Surprisingly, knocking down of Cdt2 also reduced PCNA monoubiquitination to levels observed with the depletion of Rad18 without affecting the basal level of Rad18. These results suggest that Cdt2 regulate the basal level of PCNA monoubiquitination.

Exogenous Cdt2 rescues the effect of si-Cdt2

The last experiment was done in U2OS cells and not in MCF7 cells, because we wanted to use a cell-line where we could definitively rule out the possibility that the reduction of PCNA monoubiquitination seen after si-Cdt2 was due to off-target activity, We had U2OS cell stably transfected with and expressing exogenous siRNA-resistant Cdt2 and could use these cells to test whether the exogenous Cdt2 can restore PCNA monoubiquitination in the absence of endogenous Cdt2. We had U2OS cell which express either wild type Cdt2 or Cdt2 protein deficient in binding DDB1 (Cdt2-R246A) Since one Cdt2 siRNA (siCdt2-1) is targeting ORF, both endogenous and exogenous Cdt2 were depleted. In contrast, another siRNA targets the 3'-UTR of Cdt2 gene and affects only endogenous Cdt2. Indeed, si-Cdt2-2(targeting 3'-UTR) did not decrease PCNA monoubiquitination in cells expressing the exogenous wild type Cdt2 (Fig.

3B, lane 4). On the other hand, the mutant Cdt2-R246A could not restore PCNA monoubiquitination in cells transfected with siCdt2-2 (lane 8). These results demonstrate that Cdt2 knockdown specifically decreases PCNA monoubiquitination, and also that Cdt2 has to bind to DDB1 (and through it to the rest of the CRL4 complex) to monoubiqutinate PCNA.

CRL4^{Cdt2} complex promotes PCNA-dependent translesion DNA synthesis (TLS)

PCNA monoubiquitination plays a significant role in TLS by attracting the error-prone translesion polymerases to the site of DNA damage(Andersen et al., 2008). Because, CRL4Cdt2 promotes PCNA monoubiquitination under basal conditions, we tested whether such an activity impacts TLS in the absence of extrinsic DNA damage. We measured the TLS activity (Parris and Seidman, 1992) by measuring the mutation frequency in a supF gene (in a shuttle vector) subjected to UV-induced DNA damage before its introduction into the cells. Error-prone TLS activity will mutate the supF gene, which is scored by a blue-white colony screen after recovering the supF shuttle vector from the mammalian cells to transfect into bacteria. To increase the sensitivity of the assay, we performed these experiments in 293T human embryonic kidney epithelial cells, where the transfected supF reporter gene could replicate (they do not replicate in any of the breast cancer cells. In addition, we depleted the 293T cells of the high fidelity TLS enzyme, DNA polymerase eta (pol eta) (Huang et al., 2006). Consistent with previous reports, cells depleted of USP1 showed a five-fold increase in mutation frequency compared to cells with USP1 (Figure 4A). Sequencing of the mutant supF genes, revealed a mutation spectrum consistent with error prone TLS (Supplemental Table 1). Significantly, the depletion of Cdt2 from these cells reduced the mutation frequency, indicating that the reduction in monoubiquitinated PCNA (Figure 1D) inhibits TLS activity and mutation fixation in vivo. Similar results were obtained when 293T cells were depleted of the other components of the CRL4Cdt2, DDB1 and Cul4A/B, but not upon depletion of Cul1 or DDB2 (Figure 4B). These results demonstrate that the inhibition of TLS activity was a consequence of the specific inactivation of CRL4Cdt2 complex.

Key accomplishments:

- Obtained and validated reagents for an inducible Cdt1-expression-system
- Identified appropriate dose of MTX for testing gene amplification
- Identified that PCNA monoubiquitination is induced by CRL4Cdt2 E3 ligase complex in MCF7 breast cancer cell lines.
- Identified that the depletion of Cdt2 reduces the steady-state level of PCNA mono-ubiquitination.
- Identified that exogenous Cdt2 rescues the effect of si-Cdt2.
- Identified that CRL4^{Cdt2} complex promotes PCNA-dependent translesion DNA synthesis and mutation fixation.

Reportable Outcomes:

Conclusions:

Expression level of Cdt1 was induced by Doxcycline in a dose-dependent manner.

By using Doxcycline, we confirmed inducible Cdt1 expression. Using the Doxcycline system, I will analyze DNA replication in breast cancer cells using FACS after inducing

differing levels of Cdt1. From these results, I will estimate the highest and lowest dose of Doxcycline for testing whether moderate over expression of Cdt1 promotes DNA amplification.

CRL4Cdt2 E3 ubiquitin ligase monoubiquitinated PCNA to promoted translesion DNA synthesis and mutation fixation.

Additionally, we have identified a novel target of CRL4 complex. Cdt2 depletion reduced the basal level of monoubiquitinated PCNA and decreased the activity of translesion DNA synthesis. Since the monoubiquitination of PCNA is a key regulator for (a) increasing the tolerance for DNA damage reagents and (b) creation (fixation) of point mutations after DNA damage, it is possible that depletion or inhibition of Cdt2 enhances the effect of anti-cancer drugs. Furthermore, since TLS increases mutation frequency, it is tempting to speculate that the over-expression of Cul4A or Cdt2 observed in some breast cancer (Pan et al., 2006; Schindl et al., 2007) may promote malignancy by enhancing PCNA-dependent and TLS-mediated mutagenesis.

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NAME	POSITION/TITLE
Kenta Terai	**Research Associate**

EDUCATION AND TRAINING			
Institution And Location	Degree (If Applicable)	Year Conferred	Field Of Study
Hokkaido University, School of Medicine (Japan)	**MD**	2002	
Osaka University, Graduate School of Medicine (Japan)	**PhD**	2006	Medicine

RESEARCH AND PROFESSIONAL EXPERIENCE

Concluding with your current position, list chronologically previous employment, experience and honors. Also list in chronological order the titles and complete references to all publications during the past three years and to representative earlier publications pertinent to this application.

Research experience

From 01/04/2002 to 31/03/2006 : Osaka University (Suita, Japan) Graduate student Signal Transduction (Michiyuki Matsuda)

From 01/04/2006 to 31/05/2006 : Kyoto University (Kyoto, Japan) Research Associate Signal Transduction (Michiyuki Matsuda)

From 01/06/2006 to : University of Virginia (Charlottesville, U.S.) Research Associate Biochemistry & Molecular Genetics (Anindya Dutta)

Awards

Uehara Foundation Fellowship, Jan 2007-Dec 2007

DoD Era of Hope Postdoctoral Fellowship, Jun 2008-

List of publications

Kurokawa K, Takaya A, **Terai K**, Fujioka A, Matsuda M. Visualizing the signal transduction pathways in living cells with GFP-based FRET probes. Acta Histochemica et Cytochemica 2004, 34, 347-355.

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Fig. 1A

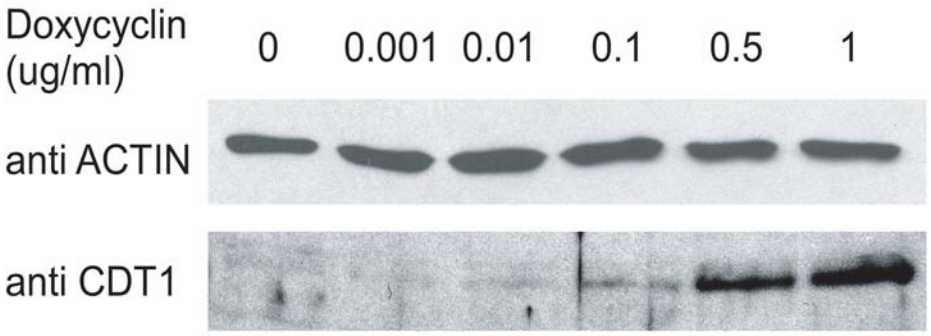
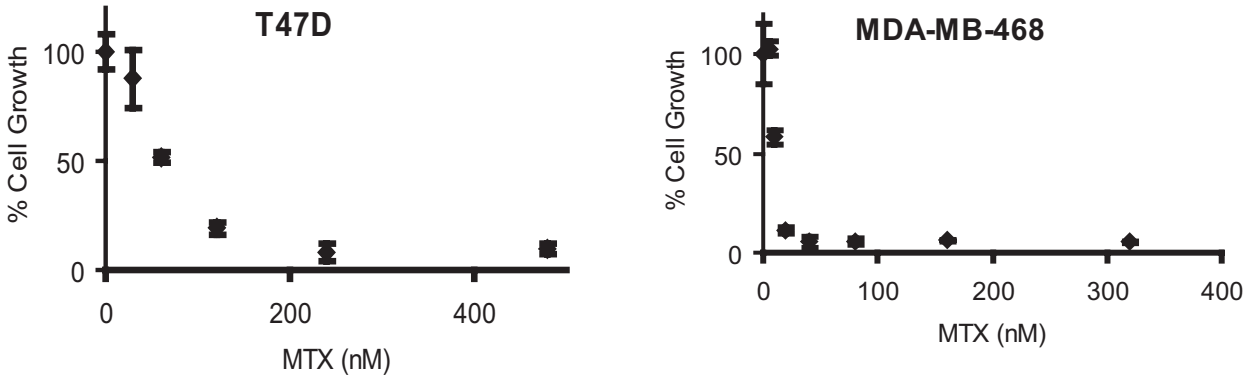
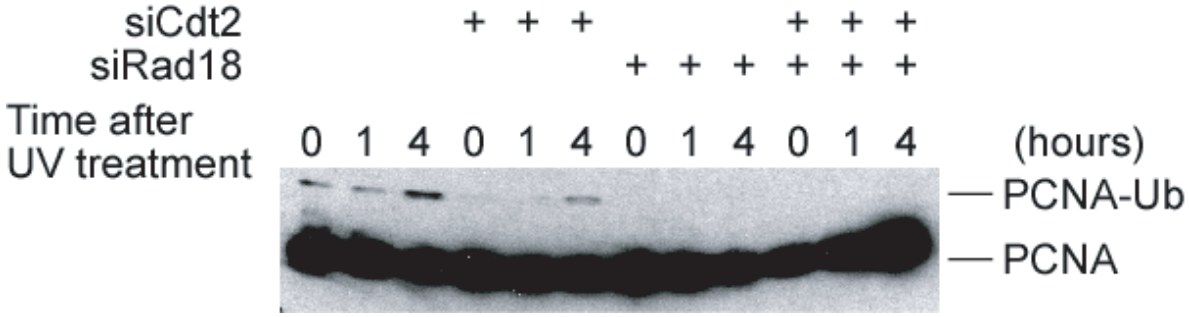


Fig. 1B



(A) U2OS-TetON-hCdt1 cells were treated with a different dose of doxycycline. After two days, cells were lysised and analyzed with Cdt1 antibody. (B) T47D and MDA-MB-468 cell lines were treated with indicated dose of MTX. Five days after treatment, nubers of cells were counted.

Fig. 2



MCF7 cells were transfected siRNA of Cdt2, Rad18, or Cdt2 and Rad18. After two days, cells were irradiated with UV at 50 J/m^2 and lysised indicated time points. Cell lysates were probed with PCNA antibody.

Fig. 3A

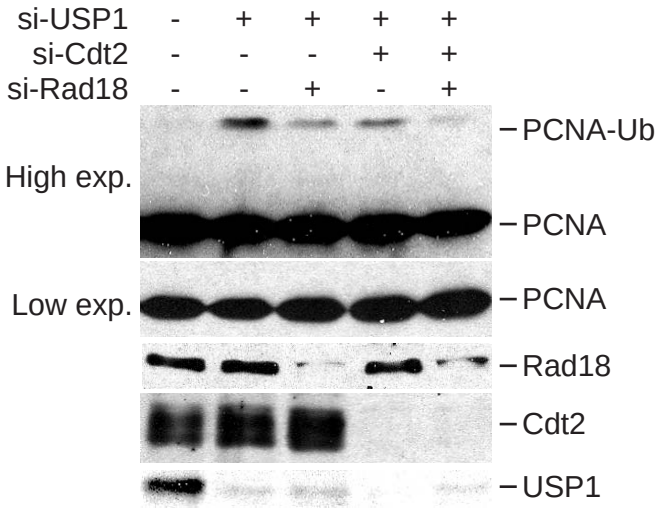
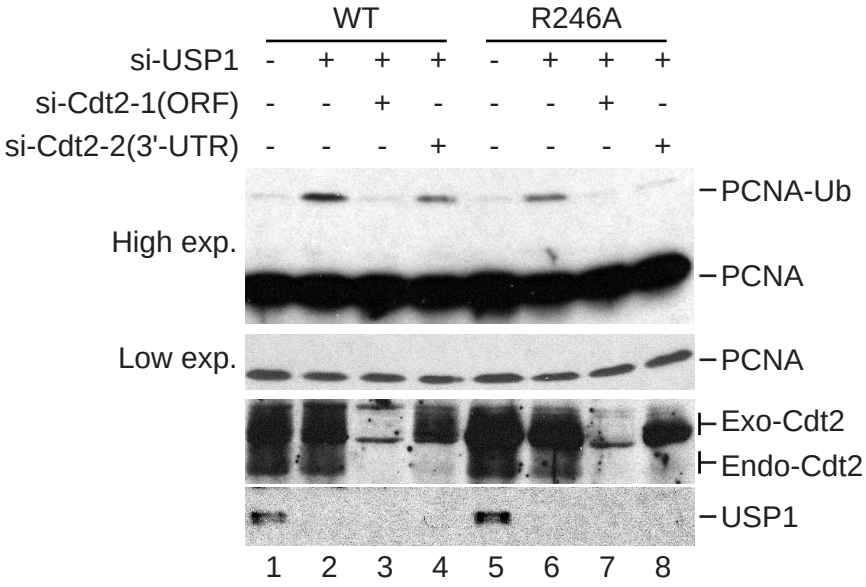


Fig. 3B



(A) U2OS cells ware transfected with the indicated siRNA. After 2 days, cells were analyzed by immunoblotting with anti-PCNA, anti-Rad18, anti-Cdt2, or anti-USP1 antibody. (B) U2OS cells stably expressing either wild type and flag-tagged Cdt2 or Cdt2 mutant protein (Cdt2-R246A) were transfected with si-Cdt2-1 (targeting the ORF) or with si-Cdt2-2 (targeting the 3'-UTR). Where indicated cells were also transfected with si-USP1. Two days after transfections, cells were harvested and the cell lysates analyzed by immunoblotting with anti-PCNA, anti-Cdt2, or anti-USP1 antibody. The anti-Cdt2 blot shows endogenous and exogenous Cdt2.

Fig. 4A

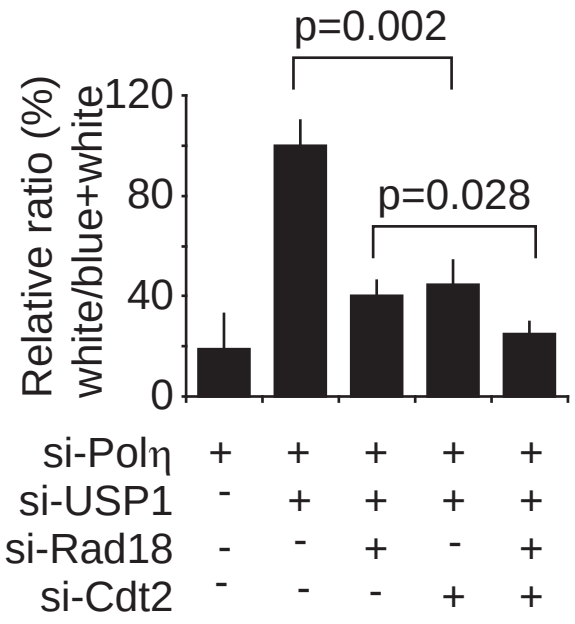
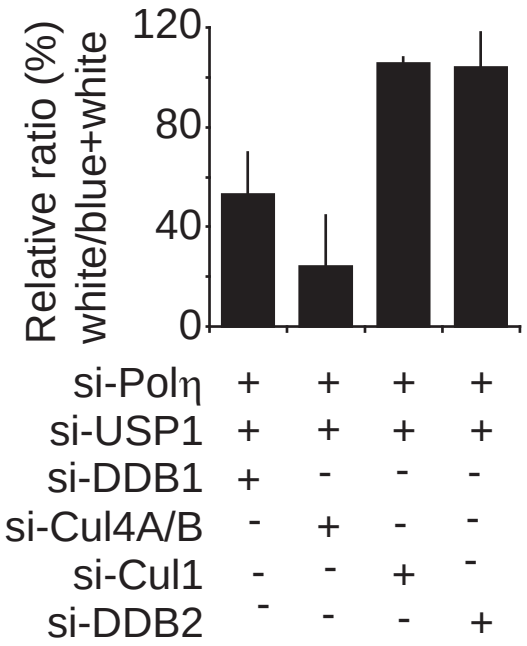


Fig. 4B



(A and B) 293T cells were transfected with the indicated siRNA. Twenty-four hours after incubation, UV-treated reporter plasmid was introduced into the 293T cells. Forty-eight hours after this, plasmids were purified from cells and analyzed for mutation frequency expressed as ratio of white to total colonies. The ratio seen after si-USP1+ si-pol eta is shown as 100%. Mean and S.D. of 3 experiments